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Vaccination with syngeneic monoclonal anti-idiotype protects against a tumour challenge

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SUMMARY

Rat × rat hybridomas secreting monoclonal anti-idiotypic antibodies have been prepared from Hooded rats immunized with two tumour-reactive, syngeneic monoclonal antibodies 11/160 and M10/76 (specific, respectively, for the Hooded rat sarcomata HSN and MC24). The hybridomas were selected on the basis that the secreted antibodies competed with antigen for binding to the immunizing idiotype. One monoclonal anti-idiotype (HIM/1/230, γ2a isotype) that recognizes an antigen-binding site idiotope of antibody 11/160 has been found to substitute for antigen. Hooded rats vaccinated by three challenges with HIM/1/230 (i) produce serum Ab_3 that is indistinguishable in antigen specificity from the 11/160 Ab_1, and (ii) show reduced tumour take following an i.v. challenge with 10^6 HSN cells. The response to vaccination with anti-idiotype was both qualitatively and quantitatively dependent on the mode of immunization. High titre 11/160-like Ab_3 was generated only when the vaccine contained Freund's adjuvant, whereas resistance to tumour challenge was found only in animals vaccinated with anti-idiotype in the absence of adjuvant.

INTRODUCTION

There is considerable interest in the possibility of using anti-idiotypic antibodies for modulation of immune responses: in particular, the use of internal image anti-idiotype which can susbstitute for antigen and induce protective immunity. Anti-idiotypes thought to mimic antigen have been generated in several systems, and their administration *in vivo* has been shown to either enhance or suppress a relevant immune response (Sharp *et al.*, 1984; Stein & Soderstrom, 1984; Uytdehaag & Osterhaus, 1985; Flood *et al.*, 1980). We are interested in the possible use of such antibodies for the treatment of cancer, and are investigating this aspect using two non-cross-reactive, chemically induced rat sarcomata. These tumours elicit specific antibody responses in syngeneic hosts, and two IgG2b secreting hybridomas (11/160 and M10/76), the antibodies of which are specific to the HSN and MC24 tumour, respectively, have been prepared using spleens of tumour bearers (North *et al.*, 1982). In this communication, we report the preparation of syngeneic monoclonal anti-idiotypic antibodies that mimic the specific antigens present on the surfaces of these tumours. We show that when one of these antibodies (HIM/1/230) was injected into naive rats, it induced a tumour-specific humoral response and gave protection against a subsequent challenge with the HSN tumour.

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MATERIALS AND METHODS

Immunization with idiotype

Ten- to twelve-week-old female CBH rats were taken from our isolator-maintained colony and immunized three times (twice via the Peyer's patches and once i.p., Dean *et al.*, 1986) at 14-day intervals with 100-500 μg of affinity-purified monoclonal antibodies 11/160 or M10/76 (North *et al.*, 1982). The initial immunization was given in complete Freund's adjuvant (CFA, Difco Laboratories, Detroit, MI) the remainder in incomplete Freund's adjuvant (IFA). The rats were bled just before each immunization and 7 days after the last injection. All sera were stored at $-20°$.

Immunization with anti-idiotype HIM/1/230

Ten- to twelve-week-old female CBH rats were immunized at five sites (4 × s.c. and 1 × i.p.) with 100 μg, 1 μg or 10 ng of affinity-purified HIM/1/230 in CFA or phospate-buffered saline (PBS) and then boosted 14 and 28 days later with the same doses of antibody in IFA or PBS. Antibody HIM/1/230 was prepared from either ascites grown in nude rats or hybridoma culture supernatant. The antibody was isolated from ascitic fluid by $(NH_4)_2SO_4$ precipitation and chromatography on Whatman DE52 cellulose (Whatman Ltd, Maidstone, Kent) then purified by binding to 11/160 linked to Sepharose 4B (Pharmacia Ltd, Uppsala, Sweden) and elution with 3 M KSCN. HIM/1/230 was isolated from culture supernatant by affinity chromatography on 11/160-Sepharose 4B.

Hybridoma production

The protocol for preparation of rat × rat hybridomas using cells from the mesenteric nodes or spleens of immune rats and the rat myeloma Y3 Ag.1.2.3 (Galfré, Milstein & Wright, 1979) has been described previously (Dean *et al.*, 1984).

Screening for specific antibodies

Cell-binding antibodies were detected using monolayers of HSN or MC24 cells grown in Nunc 96-well plates (Gibco Europe Ltd, Uxbridge, Middlesex) as described previously (North *et al.*, 1982). Bound antibodies and their isotypes were determined using ^{125}iodine-labelled affinity-purified sheep, rabbit or goat antibodies to rat F(ab′)$_2$, γ_1, γ_2, α or μ.

Competitive radioimmunoassays were used to detect specific anti-idiotype. Dilutions of rat sera or monoclonal antibodies were made in TC199 medium containing 5% fetal bovine serum and 20 mM HEPES. Fifty microlitres of these dilutions were mixed with an equal volume of ^{125}I-labelled 11/160 or M10/76 (2×10^4 c.p.m. of a stock labelled to a specific activity of 10 μCi/μg by the chloramine T procedure of Greenwood, Hunter & Glover, 1963) and added to target cells grown to confluence in Nunc 96-well plates. After incubation for 1 hr at 18–20°, the plates were washed three times with medium, then the cells were lysed and the bound radioactivity determined.

Specific idiotype and anti-idiotype in sera were also detected using polyvinyl microtitre plates (Dynatech Ltd, Billingshurst, Sussex) that had been coated by incubation overnight at 4° with 50 μl/well of F(ab′)$_2$ 11/160 or F(ab′)$_2$ HIM/1/230 (1 μg/ml in 0·1 M phosphate buffer, pH 8·2) and then blocked by incubation for 1 hr at 37° in PBS containing 0·5% bovine serum albumin (PBS–BSA). Serial dilutions of sera or monoclonal antibodies were added to the plates (50 μl/well) and after 1 hr incubation at 18–20° the plates were washed three times and the quantity of bound antibody determined using ^{125}I-sheep anti-rat IgG.

Lung colonization assay

HSN cells were removed from a subconfluent monolayer by incubation in PBS containing 0·04% EDTA for 15 min at 37°. One million cells contained in 0·2 ml PBS were injected via the jugular vein into control or vaccinated female rats (see Results). The rats were killed 28 days later and their lungs removed, fixed for 24 hr in Bouin's fluid, weighed, and the number of surface colonies counted.

RESULTS

Production of anti-idiotype antibodies

Hooded rats immunized via their Peyer's patches with the monoclonal antibodies 11/160 or M10/76 had specific anti-idiotype in their sera as judged by the results of two binding assays using HSN or MC24 monolayers as target antigen. The first, a competitive radioimmunoassay (RIA), showed that binding of ^{125}I-labelled specific Ab_1 was inhibited by sera from immunized animals (Table 1, Fig. 1) but not by sera from control animals. The second test, an indirect binding assay using ^{125}I-sheep anti-rat F(ab′)$_2$, showed that the competing antibodies (Ab_2) present in immune sera did not bind to target cells (Table 1). These results suggest that serum Ab_2 from immune rats included paratope-related anti-idiotypes which competed with antigen for the binding site of Ab_1.

Preparation of monoclonal anti-idiotype

Rats that had been immunized with idiotype via the Peyer's patches were used for the preparation of hybridomas using both mesenteric nodes and spleens as sources of lymphoid cells. The fusions were screened for antibodies that inhibited binding of Ab_1 to target cells, i.e. antibodies that were likely to be anti-paratopic. In the 10 fusions performed, 25 clones of this type

Table 1. Reactivity of sera from CBH rats hyperimmunized with syngeneic monoclonal antibodies as assayed by competition for binding of Ab* or direct binding to target antigen†

	C.p.m. ^{125}I M10/76 bound to MC24 cells*	C.p.m. ^{125}I sh/rat F(ab′)$_2$ bound to MC24†	C.p.m. ^{125}I 11/160 bound to HSN*	C.p.m. ^{125}I sh/rat F(ab′)$_2$ bound to HSN†
(a) *Animals immunized with M10/76*				
None	1719	—	2015	—
Normal serum	1226	200	1607	—
Rat 1 serum	161	—	—	—
Rat 2 serum	27	—	—	—
Rat 3 serum	199	141	1895	—
Rat 4 serum	228	66	1571	—
Rat 5 serum	726	75	1565	—
M10/76	22	1836	—	—
11/160	1593	—	—	—
(b) *Animals immunized with 11/160*				
None	2270	—	982	—
Normal serum	2156	—	601	918
Rat 6 serum	1820	—	14	661
Rat 7 serum	2060	—	19	764
Rat 8 serum	1267	—	11	736
Rat 9 serum	—	—	14	—
M10/76 (1 mg/ml)	—	—	—	112
11/160 (1 mg/ml)	—	—	26	3940

Sera were tested at a dilution of 1/8.
(—) Not tested.

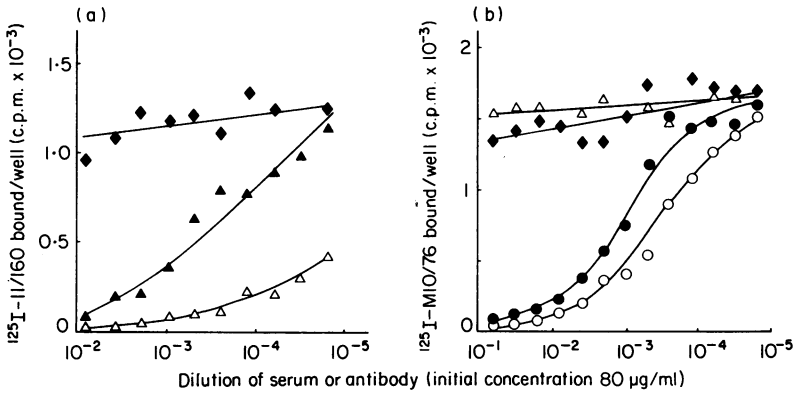

Figure 1. Humoral response of Hooded rats immunized with antibodies 11/160 or M10/76. Inhibition of binding of (a) ^{125}I-11/160 to HSN cells and (b) ^{125}I-M10/76 to MC24 cells by: (▲) serum from a rat immunized with 11/160; (●) serum from rat immunized with M10/76; (♦) pooled normal rat serum (NRS); (△) affinity-purified 11/160; (○) affinity-purified M10/76.

were recovered that secreted antibodies directed against the 11/160 idiotype, and a further eight that recognized the M10/76 idiotype (Table 2). Although large numbers of hybridomas (> 300 per fusion) were obtained when either spleen or mesenteric lymph node cells were used for fusion, hybridomas secreting specific anti-idiotype were found only when cells from mesenteric nodes were used as the lymphoid source. Twenty-eight of the specific hybridomas secreted IgG antibodies, and 20 of these were of the IgG1 isotype. The predominance of IgG1-secreting hybridomas was surprising, but this finding is in accord with those of others using rodents (Rousseaux-Prevost *et al.*, 1983; Sakato & Eisen, 1975; Fricke, Bridges & Lynch, 1977).

Three of the monoclonal antibodies with specificity for 11/160 but of differing isotype (HIM/1/230, γ2a; HIM/3/32, γ1; HIM/4/20, α) were purified from culture supernatants by affinity chromatography on Sepharose-linked 11/160. All were found to bind to 11/160 F(ab')$_2$-coated PVC flexiplates (data not shown), but they varied in their ability to displace 11/160 from target HSN antigen. HIM/1/230 displaced the highest percentage of radiolabelled 11/160 specifically bound to target cells, 67% after 1 hr incubation, whereas HIM/3/32 and HIM/4/20 displaced only 53% and 35% of the bound immunoglobulin, respectively. From these data, it was concluded that the IgG2a antibody HIM/1/230 was the most active, and it was selected for studies *in vivo*.

Vaccination with HIM/1/230 elicits an 11/160-like Ab$_3$ response

In order to discover if the monoclonal Ab$_2$, HIM/1/230, was directed against the paratope of 11/160, CBH rats were inoculated with 100 µg doses of HIM/1/230 and the sera examined for Ab$_3$ showing similar specificity to 11/160. The results of binding and competitive RIAs showed that sera from animals immunized with HIM/1/230 in Freund's adjuvant contained Ab$_3$ that both competed with 11/160 for, and bound directly to, target antigen on HSN cells (Fig. 2). Further data (not shown) indicated that the antibody response in these animals was polyclonal and included specific antibodies of the γ2a, γ2b, and γ1 isotypes. We conclude that HIM/1/230 is a paratopic anti-idiotype that mimics the specific antigen of the HSN tumour. These results confirmed our expectation that, in an all-syngeneic system, the paratope of an antibody molecule represents a major idiotypic determinant recognized by the immune system.

Table 2. Hybridomas obtained from fusions of Y3 Ag1.2.3. with cells from mesenteric nodes or spleens of rats immunized via the Peyer's patches

Fusion no.	Source of lymphocytes	Wells +ve/ total no. wells	Isotypes of cloned hybridomas: IgM	IgG1	IgG2	IgA	Other
Rats immunized with 11/160							
HIM 1	Node	9/384	—	—	1	—	—
HIM 2	Node	0/96	—	—	—	—	—
HIM 3	Node	18/96	1	7	5	—	2
HIM 4	Node	14/96	1	4	2	1	—
HIM 5	Node	1/96	—	1	—	—	
HIS 1	Spleen	0/96	—	—	—	—	—
HIS 2	Spleen	0/96	—	—	—	—	—
Rats immunized with M10/76							
MIN 1	Node	9/96	—	6	—	—	—
MIN 2	Node	8/96	—	2	—	—	—
MIS 1	Spleen	0/96	—	—	—	—	—

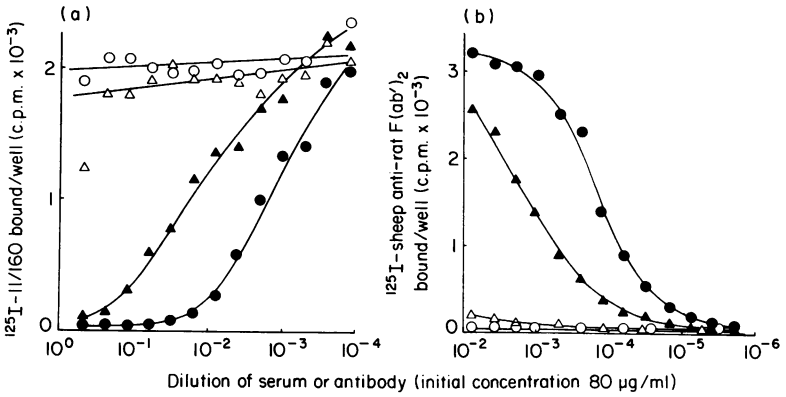

Figure 2. Specificity of Ab_3 response in rats immunized with anti-idiotype, HIM/1/230. (a) Inhibition of ^{125}I-11/160 binding to HSN cells and (b) specific antibody binding to HSN cells using: (▲) serum from a rat immunized with HIM/1/230; (△) pooled NRS; (●) affinity-purified 11/160; (○) affinity-purified M10/76.

Vaccination with HIM/1/230 protects against a tumour challenge

In order to investigate if vaccination with anti-idiotype modified the response of the host to HSN tumour cells, rats were vaccinated with varying doses of HIM/1/230 (100 μg, 1 μg or 10 ng/immunization) contained in adjuvant or PBS, and their sera examined for circulating specific antibody before challenge, by intravenous injection, with live tumour cells. The results of RIAs used to measure both idiotype and anti-idiotype levels in the sera of animals vaccinated with the highest dose of anti-idiotype are shown in Fig. 3. The results of a lung colonization assay used to assess the effect of vaccination on a subsequent tumour challenge are presented in Table 3.

In agreement with earlier data, the rats vaccinated three times with 100 μg of HIM/1/230 in the presence of Freund's adjuvant responded by production of high titre serum antibodies bearing the 11/160 idiotype [as judged by competitive assays on target cells and by binding to F(ab')$_2$ HIM/1/230]. The quantity of HIM/1/230-like antibody in sera of these animals did not increase with time, and the serum level declined after each vaccination at a rate consistent with the normal turnover of rat γ2a immunoglobulins (Peppard & Orlans, 1980). Rats vaccinated with 100 μg of HIM/1/230 in PBS alone, however, showed increased levels of Ab_2-like antibody after challenge,

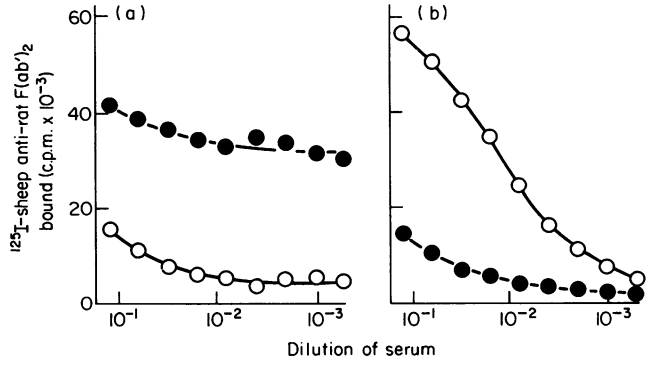

Figure 3. Influence of adjuvant on the Ab_3 response of rats immunized with HIM/1/230 either (●) emulsified in Freund's adjuvant or (○) in PBS. Specific idiotype (a) or anti-idiotype (b) in serum was determined by binding F(ab')$_2$ HIM/1/230 or F(ab')$_2$ 11/160.

whereas the level of 11/160-like antibody in the sera of these animals, although low, showed that it is possible to generate an Ab_3 response in the absence of adjuvant. It was not clear from these experiments if the increased serum levels of HIM/1/230-like antibody following vaccination in PBS alone was due to *de novo* synthesis or to accumulation of material injected s.c. and i.p. because of a longer life-time of the Ab_2 in these animals. Tests using ^{125}I-HIM/1/230 injected in a similar manner indicated that a slow clearance from blood was responsible for the build-up in serum of antibodies with Ab_2 activity. Of the animals vaccinated with lower doses of HIM/1/230, only those given 1 μg in adjuvant had 11/160-like Ab_3 in sera that could be detected by competitive assay on HSN cells, and the titres were correspondingly lower. Using the more sensitive assay of binding to F(ab')$_2$ HIM/1/230-coated plates, 11/160-like antibodies could be detected also in the sera of animals that had been given 10 ng of HIM/1/230 in adjuvant, but no antibodies of this type were found in rats vaccinated with either 1 μg or 10 ng or anti-idiotype in PBS alone. Increased levels of HIM/1/230-like antibodies were found only in the sera of animals that had been challenged with the highest dose of anti-idiotype.

In the lung colonization assay we were surprised to find that vaccination in the presence of adjuvant did not lead to a reduction in either the number of colonies formed or lung weights, even though high titres of 11/160-like antibody were present in serum. In contrast, the rats vaccinated with 100 μg of anti-idiotype in PBS showed a highly significant reduction in both numbers of lung colonies ($P = < 0{\cdot}01$) and lung weights ($P = < 0{\cdot}01$), indicating that this treatment had afforded the animals some protection against intravenous challenge with a large bolus of tumour cells. Although the rats that had been immunized with the lower doses of anti-idiotype showed no reduction in lung weights compared to controls, there was a significant ($P = < 0{\cdot}05$) reduction in the numbers of surface colonies present.

DISCUSSION

Our results show that the protocol used for immunizing syngeneic animals via the Peyer's patches provides a reliable method for generating paratopic anti-idiotypes that can be isolated subsequently as monoclonal antibodies using hybri-

Table 3. Influence of vaccination with HIM/1/230 on the number of surface colonies formed on and weights of lungs of CBH rats challenged i.v. with 10^6 HSN cells

	Dose/animal HIM/1/230							
	0		10 ng		1 μg		100 μg	
Treatment	Colonies	Wt (g)	Colonies	Wt (g)	Colonies	Wt (g)	Colonies	Wt (g)
Control untreated	No tumour challenge	1·2						
PBS + HIM/1/230	103	2·8	63	2·5	42	1·6	4	1·6
	51	1·8	71	2·6	26	1·8	3	1·4
	118	2·7	31	2·3	43	2·4	27	1·8
	103	2·4	48	1·75	76	2·4	34	1·7
	178	3·3					14	1·9
Mean	110·6	2·6	53·3*	2·3	46·8*	2·05	16·4†	1·68†
(SD)	(45·5)	(0·55)	(17·6)	(0·35)	(21·0)	(0·41)	(13·8)	(0·19)
Adjuvant + HIM/1/230			150	8·7	53	2·2	94	2·3
			112	3·4	83	2·4	74	2·4
			141	4·8	78	2·3	114	3·1
			154	2·6	143	3·6	116	3·7
			81	2·2	29	2·1	70	7·9
Mean			127·6	4·34	77·2	2·52	113·6	3·88
(SD)			(30·8)	(2·63)	(42·6)	(0·61)	(35·8)	(2·32)

Probability of no difference compared to untreated rats determined by *t*-test: $*P < 0.05$; $\dagger P < 0.01$.

doma technology. This strategy may prove useful for the preparation of rodent monoclonal anti-idiotype for use in clinical applications. The finding that vaccination with monoclonal Ab_2 can lead to a reduction in tumour take and elicits polyclonal antibodies of identical specificity to those found in tumour-bearer serum (Eccles *et al.*, 1979) is of some interest. The results are consistent with the hypothesis that, with the HSN tumour, the 11/160 antigen is the determinant responsible not only for these effects but also for the resistance to secondary challenge that is seen during tumour growth (concomitant immunity) and after removal of the primary tumour (secondary graft rejection) (Klein *et al.*, 1960; Mikulska, Smith & Alexander, 1966).

The failure of animals with high serum titres of specific Ab_3 to reject a tumour challenge may indicate that antibody alone is unable to confer immunity, but the influence of adjuvant on these processes needs to be investigated. It is not clear whether the immune responses we have observed were associated with an alteration in the clonal expression of idiotype on B and T cells, or if other factors were involved, e.g. mode of antigen presentation by accessory cells. The influence on these processes of the isotype of the anti-idiotype is currently under investigation. Also, the importance of Ab_2-like antibodies in the circulation of those animals that were resistant to a subsequent tumour challenge must not be overlooked. Indeed, Flood *et al.* (1980) have suggested that autologous anti-idiotype may regulate tumour-specific immunity, and in cancer patients there is evidence also to show that autologous serum anti-idiotype may facilitate tumour regression (Koprowski *et al.*, 1984).

These results point to the importance of the idiotype network (Jerne, 1974; Urbain *et al.*, 1979) in the response to tumour antigens of man and experimental animals. It may be possible, therefore, to modify the immune response in malignant disease with therapeutic results. In experimental systems anti-idiotype antibodies have been found to induce an immune response and to influence tumour growth (Forstrom *et al.*, 1983; Nepom *et al.*, 1984; Gorczynski *et al.*, 1984; Kennedy *et al.*, 1985; Stevenson, Elliott & Stevenson, 1979). In addition, the regression of lymphoid tumours has been reported in patients treated with murine monoclonal anti-idiotypic antibodies (Miller *et al.*, 1982). Our results show that it is possible to vaccinate rats against a subsequent challenge with syngeneic tumour cells. The finding that successful vaccination against the HSN tumour did not require the production of high titres of specific Ab_3 in serum leads us to suspect that the immunity induced was cell mediated, and this aspect is currently under investigation.

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